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Landscape Phage Probes for *Salmonella typhimurium*

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Non-standard abbreviations

AU—absorbance units; CFU--colony forming units; DOC—deoxycholate; FACS—fluorescence activated cell sorting; OD –optical density; PCR—polymerase chain reaction; RT--room temperature, SEM—scanning electron microscopy; TEM—transmission electron microscopy; vir—virions.

ABSTRACT

We selected from a landscape phage library probes that bind preferentially *Salmonella typhimurium* cells compared with other *Enterobacteriaceae*. The specificity of the phage probes for *Salmonella typhimurium* was analyzed by the phage-capture test, the enzyme-linked immunosorbent assay (ELISA) and the precipitation test. Interaction of representative probes with *Salmonella typhimurium* was characterized by fluorescence-activated cell sorting (FACS), and fluorescent, optical and electron microscopy. The results show that the landscape phage library is a rich source of highly specific and robust probes for *Salmonella typhimurium* suitable for long-term use in continuous monitoring devices and biosorbents.

THE VIEWS EXPRESSED IN THIS ARTICLE ARE THOSE OF THE AUTHOR AND DO NOT REFLECT THE OFFICIAL POLICY OR POSITION OF THE UNITED STATES AIR FORCE, DEPARTMENT OF DEFENSE, OR THE U.S. GOVERNMENT.

1. INTRODUCTION

Screening of potential molecular probes for bacterial identification has long been a central tenet of bacteriology, dating back nearly 300 years to Von Leeuwenhoek, who first attempted visualization of bacteria using naturally colored agents such as beet juice (Wainwright and Lederberg, 1992). Later, Koch developed a heat-mediated methylene blue staining process for the identification of tubercle bacillus, while Hans Christian Gram developed a procedure for the differentiation of bacteria based on cell wall components staining (Donnelly, 1962) that is still widely used today. Since then, numerous molecular recognition methods have been elaborated for detection of bacteria, most attempting to incorporate speed and simplicity (Madonna et al., 2001; Mansfield and Forsythe, 2000; Meckes and MacDonald, 2003), as reviewed in Petrenko and Sorokulova, 2004. Simplified, rapid identification is especially important for pathogenic food-borne bacteria. Among these, *Salmonella typhimurium* (*S. typhimurium*) ranks as a leading cause of bacterial food-borne illnesses in the United States (Campbell et al., 2001).

Classical *S. typhimurium* identification is based on amplification of cells on selective media (Dusch and Altwegg, 1995). However, this technique may take as many as 4-5 days to confirm identity. More advanced methods for routine *S. typhimurium* detection include polymerase chain reaction (PCR) (Kumar et al., 2003; Peng and Shelef, 2001; Tseng et al., 1997), DNA hybridization (Meckes and MacDonald, 2003; Miyamoto et al., 1998; Nastasi et al., 1999), enzyme-linked immunosorbent assay (ELISA) (Tapchaisri et al., 1999) and biosensor-based assays (Kramer and Lim, 2004; Muhammad-Tahir and Alocilja, 2003; Olsen et al., 2003; Taitt et al., 2004) These modern methods offer many potential advantages for the rapid detection of microbial and viral pathogens in environmental samples and food in comparison with traditional long-term culture

methods. Despite these advantages, their applications for real time detection and environmental monitoring remain in developmental stages with significant methodological hurdles. For example, widespread use of PCR in environmental microbiology has been limited by high sample volumes compared to miniscule amplification reaction volumes and uncontrolled organic impurities and environmental inhabitants inhibiting enzymatic reactions (Lantz et al., 2000). Thus, the uses of many rapid detection techniques could be expanded if the pathogens were separated, concentrated, and purified from the sample matrix before detection reactions (Benoit and Donahue, 2003; Stevens and Jaykus, 2004). However, existing filters and absorbents are not specific and accumulate biological agents in a complex mixture with environmental inhabitants and pollutants. Isolation, concentration of the agents and their immediate detection may be performed using advanced bioselective absorbents and biosensors. The key elements of biosorbents and biosensors are affinity probes that recognize specific structural features on the threat agents' surface. The probes (usually antibodies) are immobilized on the solid matrices of biosorbents and biosensors, allowing capturing of the agents and their registration (Petrenko and Sorokulova, 2004). However, broad application of antibodies is limited because of their high production and operational costs and a low resistance to unfavorable environmental conditions (high temperature, presence of organic solvents, heavy metals, etc.). Furthermore, although the high selectivity of antibodies is beneficial for isolation and detection of individual predicted species, it hinders the revealing of unknown, emerging or intentionally modified agents. Discriminating between live and dead agents is also an unsolved challenge for the traditional antibodies technology. Therefore, there is a need for alternative robust and inexpensive affinity probes that would capture defined groups of biological threats in highly diverse environmental conditions and allow their immediate detection.

Bacteriophages (phages) are naturally evolved molecular probes for bacteria. They are intrinsically specific to host bacteria and survive in severe environmental conditions (Bennett et al., 1997; He and Pan, 1992). There were numerous attempts to use phages as diagnostic probes for *Salmonella*. In the early 80's, Hirsh and Martin (Hirsh and Martin, 1983, 1983) utilized phage F01 for the detection of *Salmonella* in milk using HPLC. The same phage was utilized (Bennett et al., 1997) as a biosorbent probe for separation, concentration and detection of *S. enteritidis* in food. Recombinant phage containing luciferase-encoding gene was used recently for the detection of *Salmonella* (Kuhn et al., 2002; Kuhn et al., 2002). These works showed a prospect of using phage as a substitute for antibody probes, however, with a limited application because of the lytic nature of the used phages.

Phage display technique is a new approach for development of phage-derived diagnostic probes [reviewed by (Petrenko and Sorokulova, 2004; Petrenko and Vodyanoy, 2003; Smith and Petrenko, 1997)]. Our studies of landscape phages—recombinant filamentous phages displaying 4,000 foreign random peptides on their surface--showed that these nonlytic secreting viruses of *Escherichia coli* are well suited for obtaining durable, specific diagnostic probes and biosorbents for various analytes (Petrenko et al., 1996). The probes are selected from the phage display libraries, which are constructed by a genetic modification of the filamentous phage fd (Smith and Petrenko, 1997). The outer coat of the phage is dominated by multiple copies of the major coat protein pVIII, which forms a tube encasing the viral DNA and comprises 87% of the phage mass (Webster, 2001). To create a landscape phage display library, degenerate synthetic oligonucleotides are spliced in-frame into the pVIII protein gene, so that the "guest" peptides encoded by the degenerate oligonucleotides are fused to the coat protein and thereby displayed as diverse "organic landscapes" on the surface of the virions (Petrenko et al., 1996; Petrenko et al., 2002). A landscape

library is a huge population of such phages, encompassing billions of clones with different surface structures and biophysical properties. The landscape phage library, f8/8, used in this work contains random octapeptides fused to all 4000 copies of the major coat protein (Petrenko et al., 1996).

Landscape phages have been shown to serve as substitutes for antibodies against various soluble and cell-displayed antigens and receptors (Legendre and Fastrez, 2002; Mount et al., 2004; Petrenko and Smith, 2000; Petrenko et al., 2002; Romanov et al., 2001; Samoylova et al., 2003; Samoylova et.al., 2004) including bacterial cells and spores (Brigati et al., 2004; Petrenko and Sorokulova, 2004). The phage probes have been used in ELISA and thickness shear mode quartz sensors to detect bacterial and mammalian antigens (Petrenko and Smith, 2000; Petrenko and Vodyanoy, 2003). Phage-derived probes inherit the extreme robustness of wild-type phage (Brigati & Petrenko, unpublished) and allow fabrication of bioselective materials by self-assemblage of phage or its composites on metal, mineral or plastic surfaces. The prospects of phage-derived probes for isolation and detection of biological agents has been illustrated recently (Brigati et al., 2004; Petrenko and Smith, 2000; Petrenko and Sorokulova, 2004; Petrenko and Vodyanoy, 2003). In our study, we used a landscape phage library f8/8 (Petrenko et al., 1996) to isolate phage clones displaying peptides capable of specific and strong binding to *S. typhimurium* [preliminary results of the study were reviewed briefly (Petrenko and Sorokulova, 2004)]. Along with other recently published data (Brigati et al., 2004), the results of this work validate the concept of landscape phages as probes for separation, concentration and detection of biological threat agents.

2. MATERIALS AND METHODS

2.1. Solutions, reagents and media.

Solutions, reagents, preparations and media are described in Table 1.

2.2. Bacteria and library.

Salmonella typhimurium ATCC 13311 was obtained from the American Type Culture Collection, Manassas, VA. *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 25933, *Enterobacter aerogenes* ATCC 13048, *Citrobacter freundii* ATCC 8090, *Shigella flexneri* ATCC 12022, *Klebsiella pneumoniae* ATCC 13882, *Yersinia enterocolitica* ATCC 9610, *Serratia marcescens* ATCC 13880, and *Pseudomonas aeruginosa* ATCC 27853 were acquired from the Auburn University culture collection (Auburn, AL). Cultures were previously confirmed for identity by biochemical, serological, and morphological tests. *E. coli* K91BlueKan (Yu and Smith, 1996) was used for propagation of phage clones. Stock organisms were maintained in NZY broth containing 20% (v/v) glycerol at – 20 ^{O}C.

The landscape phage library f8/8, containing about two billion different clones, was previously described (Petrenko et al., 1996; Petrenko et al., 2002). The library was constructed by replacing amino acids E2, G3 and D4 on every copy of the pVIII coat protein of vector f8-1 (fd-tet derivative) with eight random amino acids.

2.3. Phage growth, purification and titering

The general procedures used for recombinant phage production and analysis, including media and buffers, are detailed in "Phage Display, A Laboratory Manual" (Barbas et al., 2001). Phage were purified by double PEG precipitation as previously described (Smith and Scott, 1993; Yu and Smith, 1996). The total number of viral particles present in phage preparations was determined spectrophotometrically using the formula (Barbas et al., 2001):

virions (vir)/ml = ($A_{269} \times 6 \times 10^{16}$) /number of nucleotides in the phage genome,

where A_{269} is absorbance at 269 nm.

For the recombinant phages used in this work (9198 nucleotides), the formula:

$$\text{absorbance unit } (AU)_{269} = 6.5 \times 10^{12} \text{ vir/ml}$$

was used to determine the concentration of phage particles in a solution (physical titer). The concentration of infective phage particles (biological titer) of a phage solution was determined by infecting starved K91BlueKan cells (Barbas et al., 2001) with the phage, followed by their spreading on a tetracycline-containing agar plate. The recombinant phages carry the gene necessary for tetracycline resistance, allowing only those cells infected by phage to form colonies on the plate. The biological titer of these recombinant phages (expressed as colony forming units/ml or CFU/ml) is typically 20 times lower then the physical titer (vir/ml).

2.4. Selection of bacteria-binding phage clones

In selection procedures A and B phage particles interacted with bacterial cells *S. typhimurium* adsorbed to a solid support. In selection procedure C, complexes of phages with *S. typhimurium* were formed in solution and were separated from unbound phages by precipitation, as described below.

2.4.1. Selection procedure A

S. typhimurium was propagated in 20 ml NZY medium in a shaker-incubator (16 hours, 200 rpm, 37 °C), precipitated (5,500 rpm, 15 min, Allegra 21R Centrifuge, Beckman Coulter, rotor S4180), washed with 20 ml PBS and resuspended in 4 ml PBS. A portion of the suspension (1 ml) was spread onto an empty 35 mm Petri dish and dried overnight at 37 °C. The dish was blocked with 0.1% BSA in TBS (1 hour RT) then washed 3 times with 1 ml TBS/0.1% Tween. The f8/8 phage library (10^{11} virions in 400 µl TBS, 0.1% BSA) was added to the dish and incubated 1 hour at room temperature (RT). Non-bound phage particles were removed, and the dish was washed 6 times with 1 ml of TBS/0.1% Tween. Phages bound to *S. typhimurium* were eluted with 400 µl of elution

buffer (10 min RT). The eluate was transferred to a microcentrifuge tube and neutralized with 75 µl 1M Tris (pH 9.1). The dish was washed with 0.5 ml TBS, the washing was combined with the eluate, and concentrated by centrifugation in a Centricon unit (100 kD, Millipore Corp., Bedford, MA) to ~100 µl. Remaining cell-associated phages in the dish were recovered by lysing cells with the DOC lysis buffer (250 µl, 30 min at RT). The phages from acid elution and DOC lysate fractions were propagated separately in 20 ml NZY, containing 20 ∝g/ml tetracycline and 100 µg/ml kanamycin, purified by double PEG precipitation (Barbas et al., 2001) and suspended in 200 ∝l of TBS buffer. During the second round of selection, portions of the amplified phage preparations (100 ∝l) were added to two bacteria-coated dishes, (prepared and blocked with BSA as described above), which were incubated 1 hour at RT. The dish with phages originated from the elution fraction was washed 6 times with 1 ml of TBS/0.1% Tween, and bound phages were recovered with acid elution buffer, as described above. Another dish, with the phages originated from the DOC lysis fraction, was also treated with elution buffer, washed once with 1 ml of TBS, and the remaining phages were extracted with the DOC lysis buffer. Phages from the acid elution fraction (first dish) and the DOC lysis fraction (second dish) were amplified and purified as in the first round. Likewise, the phages selected and amplified in the previous rounds were then used as an input in each subsequent round. Following the fifth round of selection, individual phage clones were amplified and sequenced to determine the amino acid sequences of the displayed peptides, as described in subsection 2.5 below.

2.4.2. Selection procedure B

Preceding the selection of *Salmonella*-binding phages, the library was depleted of phages reacting with plastic and immobilized BSA, as follows. The primary f8/8 phage library (10^{11} virions in 400

µl of TBS/0.5% Tween/0.1% BSA) was added to an empty 35 mm plastic Petri dish and incubated 1 hour at RT on the rocker. The phage suspension was decanted, transferred to another 35 mm plastic Petri dish previously blocked with 0.1% BSA in TBS (16 hours at 4 °C) and incubated for 1 hour at RT. The depleted library was recovered from the dish and transferred to a third 35 mm plastic Petri dish coated with *S. typhimurium* and blocked with 0.1% BSA in TBS (1 hour RT), as previously outlined in subsection 2.4.1. Four proceeding rounds were performed as previously outlined (2.4.1. Selection procedure A), except washing with 0.5% Tween in TBS.

2.4.3. Selection procedure C

To avoid selection of self-precipitating phage clones, the library (10^{11} virions in 400 µl of TBS) was first incubated at 70 °C for 10 min followed by adding 40 ∝l of 5% Tween-20. The phage suspension was centrifuged at 13,000 rpm 15 min [here and later for centrifugation in microtubes we used Microfuge 18 Centrifuge (Beckman Coulter, Fullerton, CA)] before adding 400 µl *Salmonella* cells (OD_{620} 0.8). After incubation of the mixture of *S. typhimurium* with phages for 1 hour, complexes of bacteria with bound phage particles were precipitated (3,500 rpm, 10 min) and washed ten times by centrifugation (3,500 rpm, 10 min) with 1 ml TBS/0.5% Tween. Following the final wash, the precipitate of cell-bound phages was suspended in 400 µl elution buffer and incubated for10 min at RT. Cells were precipitated (3,500 rpm, 10 min), the supernatant (eluate) was recovered and neutralized with 75 µl 1 M Tris (pH 9.1), and the pellet was solubilized in 250 µl DOC lysis buffer (30 min, RT, roller). Phage from both fractions (eluate and lysate) were amplified separately in *E. coli* K91BlueKan and used as the input for two proceeding rounds of selection, carried out as previously described (cf. 2.4.1. Selection procedure A). Following the third round of

selection, individual phage clones were amplified and sequenced to determine the amino acid sequences of the displayed peptides, as described in subsection 2.5. below.

2.5. PCR and DNA sequencing

Sources of phage DNAs for PCR and sequencing were colonies of infected bacteria and solutions of purified phages. Primers f8s-20 (5′-CAAAGCCTCCGTAGCCGTTG-3′) and f8as-20 (5′-CATTCCACAGACAGCCCTCA-3′) were obtained from Integrated DNA technologies, Inc., Coralville, IA.

A sample (1μl) of the suspended colony or phage diluted 1:100 in water was added to the PCR reaction tube, containing 24 μl reagents for PCR: 10 × Mg free reaction buffer (Promega, Medison, WI, USA) (2.5 volumes); 25 mM $MgCl_2$ (Promega) (2 volumes); *Taq* DNA polymerase (5 units/∝d Promega) (0.04 volumes) ; 2.5 mM dNTPs (2 volumes); primer f8s-20 (10 pmol/μl) (0.3 volumes); primer f8as-20 (10 pmol/μl) (0.3 volumes); doubly-distilled filter-sterilized water (16.9 volumes). The PCR was carried out using GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) under the following cycling conditions: one cycle at 94 °C for 3 min; 35 cycles at 94 °C for 10 s, 46 °C for 20 s, 72 °C for 45 s; and one cycle at 72 °C for 4 min. PCR products (3 μl) were analyzed by agarose gel-electrophoresis (0.8% wt/vol agarose, Sigma, St. Louis, Mo, USA) in 4×GBB buffer. Gel was stained with SYBR Green I nucleic acid gel stain (Cambrex BioScience Rockland , Rockland, ME, USA), illuminated on the Dark Reader (Clare Chemical Research, Denver CO) and DNA bands were visualized using a Kodak EDAS 290 imaging system (Eastman Kodak Company, Rochester, NY, USA). PCR products were purified using QIAquick PCR purification Kit (QIAGEN Inc., Valencia, CA) and sequenced at the Auburn University Genomic and Sequencing Laboratory (Auburn, AL) using primer f8s-20.

2.6. Co-precipitation assay

Phage suspension (300 ∝l, 10^9 CFU/ml in TBS) heated at 70 °C for 10 min was mixed with 7.5 ∝l of 20 % Tween-20 and centrifuged at 13,000 rpm for 15 min to precipitate phage aggregates. Supernatant (100 ∝l) was added to a suspension of bacterial cells (400 ∝l, OD_{620} 0.8, TBS/0.5% Tween) and incubated 1 hour at RT on a 360° rotator (Barnstead International, Iowa, Model 400110) to affect specific phage-cell binding. Cells were precipitated (3500 rpm for 10 min), washed five times with 1 ml TBS/0.5% Tween, lysed in 50 µl DOC lysis buffer (30 min at RT) and diluted with 450 ∝l TBS. Negative controls (possessing no phage) were prepared by mixing 100 µl TBS/0.5% Tween with 400 µl aliquots of bacterial suspension. As well, a negative control (possessing no bacteria) containing only 100 µl of phage and 400 µl TBS/0.5% Tween was prepared. Phage titers were determined as described in subsection 2.3. A normalized, mean recovery (output/input × 100) percentage and deviation was calculated for three duplicates.

Formation of the complexes between phages and bacteria was described through known binding equations (Connors, 1987) with some special considerations. We assumed that one or more binding sites on the phage can be involved in binding with one or more identical complementary cell surface receptors. Since the phage-complementary surface receptors on *S. typhimurium* cells are unknown, as well as a stoichiometry of their interaction with the individual phage outer coat peptides, we described the reaction between phage particles (P) and cell receptors (S) by the equation:

$$nP + S \leftrightarrow SP_n \qquad (1)$$

where,

n is the Hill coefficient, which may correspond to the number of phage particles bound to a single cell receptor; the concentration of phage in this assay is variable, and the total concentration of the cells is kept constant ($\approx 10^8$ cells/ml).

The overall binding constant (K_b) for the reaction (1) can be defined by the law of mass action (Connors, 1987):

$$K_b = [SP_n]/([S][P]^n). \qquad (2)$$

The total number of cell recptors (Cs) is composed of free receptors and those bound with phage:

$$Cs = [S] + [SP_n]. \qquad (3)$$

Combining equations (2) and (3) yields that fraction of cell receptors complexes with phage:

$$Y = [SP_n]/Cs = K_b [P]^n / (1 + K_b [P]^n) \qquad (4)$$

The ratio of receptors bound with phage to free receptors can therefore be defined by the equation:

$$Y/(1 - Y) = K_b[P]^n. \qquad (5)$$

Taking the logarithm of both sides yields:

$$\log(Y/(1 - Y)) = \log K_b + n\log[P] \qquad (6)$$

A Hill plot of $\log(Y/(1 - Y))$ versus $\log[P]$ allows an estimate of the Hill coefficient (n) from the slope, and association binding constant (K_b) from the ordinate intercept.

The portion of the cell receptors bound with phage (Y) was determined from the experimental binding isotherm as:

$$Y = Pb/Pmax \qquad (7)$$

where Pb is a number in infectious phage particles eluted from bacterial cells; and $Pmax$ is a maximal saturating number of the bound infectious phage particles, determined by sigmoidal approximation of the binding curve.

The dissociation constant can be calculated from K_b as follows:

$$K_d = 1/K_b. \qquad (8)$$

The K_d apparent is calculated by taking the Hill coefficient into account:

$$K_{d\ (apparent)} = [K_d]^{1/n}. \quad (8)$$

2.7. Fluorescently labeled phage

Phage VTPPTQHQ (here and later the names of selected phages correspond to the sequences of the phage-harbored foreign peptides) (1.2×10^{14} phage particles) was fluorescently labeled using Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Eugene, OR) as recommended by the manufacturer and isolated by double PEG-precipitation. Phage yield (9.8×10^{13} vir) and degree of labeling (~300 dye residues per phage) were determined by measuring the phage absorbance at 269 and 494 nm (dye absorption maximum) as recommended by the manufacturer.

2.8. Binding of fluorescently labeled phage to S. typhimurium

Gradually diluted solutions of fluorescently labeled phage (100 ∝l, subsection 2.7.) were mixed with suspensions of 400 ∝l *S. typhimurium* (OD_{620} 0.8 in TBS/0.5% Tween) and rotated 1 hour at RT. The samples were centrifuged (3,500 rpm for 10 min), decanted and the pellets washed twice with TBS/0.5% Tween. Following the final wash, the pellets were resuspended in PBS and analyzed by FACS (Coulter EPICS Elite Flow Cytometer, Beckman Coulter, Fullerton, CA) and fluorescence microscopy (Nikon Eclipse E800, Melville, NY).

2.9. Electron microscopy

Phage VTPPTQHQ was biotinylated as described below. A mixture of 70 µl of EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce Biotechnology Inc., Rockford, IL) (1 mg/ml in 2 mM NaOAc pH 6) and 1 ml of the phage in PBS (6.7×10^{13} vir/ml) was incubated on the roller for 2 hours at room temperature. The reaction was quenched with 0.27 ml of 1M Tris-HCl (pH 8.9) for 1 hour at RT and the preparation was dialyzed in Slide-A-Lyzer 10K Dialysis cassette (PIERCE, Rockford, IL) against 4 × 1 liter of TBS buffer at RT. The level of phage biotinylation (478 biotin residues per

phage particle) was measured using EZ^{TM} Biotin Quantitation Kit (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's procedure. Suspension of the complexes of the biotinylated phage with *S. typhimurium* (10 ∝d), obtained as described in subsection 2.6., was loaded onto formvar/carbon-coated grid and was incubated for 20 min at RT. Excess fluid was removed by absorption to a filter paper and the grid was incubated (30 min RT) with 10 ∝l EM Streptavidin-conjugated gold particles (10 nm, BB International, Cardiff, UK), diluted 1:30 with incubation buffer. The grid was washed with incubation buffer, negatively stained (0.2% phosphotungstic acid for 5 min) and examined using Philips 301 TEM at 60 kV.

2.10. Phage capture ELISA

Wells of a 96-well ELISA dish were coated overnight at 37 °C with 100 µl suspensions of *S. typhimurium* (OD_{620} 1.0) in PBS; washed five times with TBS/0.5% Tween on an automatic plate washer; blocked with 0.1% BSA in TBS and incubated for 1 hour at RT; washed again; reacted with phage clones (5×10^{10} vir in 50 µl of binding buffer for 1 hour at RT; washed again; reacted with 45 µl of biotinylated rabbit anti fd phage IgG (2.2 ∝g/ml) for 1 hour at RT; washed again; reacted with 40 µl of APSA (2.5 ml/ml) for 1.5 hours at RT, and washed again. Wells were then filled with 90 µl of NPP substrate and read on a kinetic plate reader as previously described (Yu and Smith, 1996). The slope of color development was measured as a change in optical density per 1,000 min (mOD/min). Wild-type vector f8-5 (Petrenko et al., 2002) served as a negative control for evaluation of nonspecific background binding.

2.11. Salmonella capture ELISA

Wells of a 96-well ELISA dish were coated overnight at 4 °C with 2.7×10^{10} virions in 55 µl TBS; washed five times with TBS/0.5% Tween on a plate washer; blocked with 1% BSA in TBS and

incubated 1 hour at RT, washed and reacted with approximately 5.0×10^7 cells *S. typhimurium* in 50 ∝l TBS/0.05% Tween (OD_{620} 0.8 diluted 1:20) for 2 hours RT; washed again on a plate washer; reacted for 1 hour at RT with 45 µl *Salmonella* O Poly-1 antiserum (Becton,Dickinson and Company, Franklin Lakes, NJ) (diluted 1:500 in TBS/0.5% Tween); washed again on washer; reacted for 1 hour at RT with 40 µl AP-conjugated goat anti-rabbit antibodies (diluted 1:5000 in TBS/0.5% Tween (Jackson ImmunoResearch Laboratories Inc., West Grove, PA); washed again; and developed with NPP substrate as previously described in section 2.10. above. The slope of color development was measured as a change in optical density per 1,000 min (mOD/min). Wild-type vector f8-5 served as a negative control for evaluation of nonspecific background binding.

2.12. Binding of Salmonella to the gold-immobilized phage

A gold strip (5×3×0.05 mm) was rotated for 1 hour at RT with phage suspension (5×10^{11} vir/ml in PBS), washed 5 times with PBS to remove unbound phage and soaked in suspension of *S. typhimurium* diluted in PBS (OD_{620} 1.0) on rotator for 3 hours at RT. Subsequently, the strip was washed with doubly distilled filter-sterilized water and analyzed by optical microscopy (Mitutoyo America Corporation, Aurora IL, or Nikon, Marietta GA) and scanning electron microscopy (Zeiss DSM 940 Scanning Electron Microscope, Thornwood, NY). The samples for scanning electron microscopy (SEM) were prepared by exposure to OsO_4 for an hour, air drying for 30 minutes and sputter coating with gold according to the standard SEM procedures.

3. RESULTS

3.1. Selection of phage clones that bind to S. typhimurium

The source of probes for *S. typhimurium* was the landscape library f8/8, which contains random octapeptides fused to all 4000 copies of the major coat protein of fd-tet (Petrenko et al., 1996). We

explored three differing selection procedures outlined in Table 2. In selection procedure A, a portion of the library (2×10^9 clones, 10^{11} virions) in TBS/0.1% BSA was added to 35 mm Petri dish coated with immobilized *Salmonella*. Following incubation, non-binding phage were washed away, and specific cell-associated phage were eluted with mild acid. Residual cell-bound phage virions were recovered by lysing *Salmonella* cells with DOC buffer (Ivanenkov et al., 1999). The two fractions (eluate and lysate) containing cell-specific phage were amplified separately in *E.coli* K91BlueKan host strain in the presence of kanamycin - antibiotic that destroys any *Salmonella* cells present in the fractions, but spares the host (kan+) cells. Sub-libraries enriched for *Salmonella*-binders were used in subsequent rounds of selection. In procedure B, we used more stringent conditions for selection: the library was depleted against plastic and BSA, and incubated with bacteria in the 0.5% Tween-20-containing buffer.

Taking into account that *Salmonella* can display different receptors under differing environmental conditions (Gawande and Bhagwat, 2002), we exploited also procedure C, in which phage clones bound to suspended *Salmonella* cells were separated by centrifugation. To avoid selection of self-aggregating and precipitating phage clones, the library was first heated at 70 °C and centrifuged and then rotated with a suspension of *S. typhimurium*. Two fractions of cell-associated phages isolated by acid elution followed by lysis of cells were amplified separately in *E.coli* strain K91BlueKan and used for subsequent rounds of selection. During the selection, the relative portion of *Salmonella*-associated phage in amplified phage fractions increased from one round to another (Table 3) indicating productive selection of bacterial binders. Following the 5^{th} (A), 4^{th} (B) or 3^{rd} (C) rounds, individual phage clones were analyzed by PCR and DNA sequencing and propagated for further screening.

Identified phage borne peptides can be grouped into at least five homologous families (Table 4). Peptides isolated in procedures A and B from acid fractions demonstrate striking similarities and form the family I with a leading motif VT/SP, followed by dominant P, frequent T/S, frequent T/S/Q and frequent H. Dominant phage clone VSSNQAPP in Selection C (for simplicity, phage clones are designated by the peptide sequences they bear) is conspicuously similar to that of one phage clone VSPQSAPP from selection A and may belong to the family I or may form a discrete family. Another dominant phage DRSPSSPT, eluted from suspended bacteria by acid, belongs to the family III formed by phages eluted from immobilized bacteria with DOC buffer in procedure A. Two phages VSPPSNPS and VTPPSQHA found in DOC fraction in procedure A, and phage VTPPQQGS, found in DOC fraction in procedure B, belong to the family I and probably originated from the corresponding acid fractions that can contaminate the DOC fractions. Thus, analysis of phage sequences shows that library depletion and more stringent conditions of incubation and wash in the procedure B versus procedure A influenced essentially composition of phage in both the acid and DOC fractions. Furthermore, change of mode of separation for phage-bacterial complexes in the procedure C dramatically effected the composition of the phages isolated from the acid fraction and did not permit isolation of any phage from the lysis fraction. This may imply that substrate-immobilized bacteria, versus bacteria in suspension, display different receptors, or that multivalent binding of phage to bacteria in suspension occurs less readily then to the bacteria adsorbed to a plastic matrix.

3.2. Specificity of phage binding to S. typhimurium

We define specificity as the ability of a landscape phage, possessing specific peptide sequences, to interact with a selector bacterium displaying complementary surface receptors To determine

specificity, we compared the binding of the selected phage clones with that of wild-type phage (f8-5, see Methods) and non-related recombinant phage from the f8/8mer library.

For screening of prospective phage probes, the relative binding of the isolated phage clones to *S. typhimurium* was estimated by phage-capture ELISA and a bacteria-capture ELISA. In the phage-capture assay, selected phage clones were added to the wells of a microtiter plate coated with *S. typhimurium* cells. Following incubation to effect specific-binding between cell and phage, non-binding phage particles were washed away and bound phages were revealed using biotinylated anti-phage antibodies. As shown in Table 4, selected clones bind to *S. typhimurium* to a much greater degree, in comparison to wild-type phage. In the bacteria-capture ELISA, wells of a microtiter plate were coated with phage then incubated with *S. typhimurium*. Binding of bacteria to immobilized phage was detected with rabbit anti-*S. typhimurium* antibodies, followed by reaction with alkaline phosphatase-labeled goat anti-rabbit antibodies and *p*-nitrophenylphosphate. The majority of the isolated phage clones bound to *Salmonella* at a higher degree in comparison to the control wild-type phage. Some clones bound strongly to *S. typhimurium* in both assays, while other clones gave inconsistent results between the two assays. This is not completely unexpected because in the bacteria-capture ELISA phage are fixed to the plate and cells are captured from solution, while in the phage-capture assay cells are fixed to the plate and phages are bound from solution. Thus, in these tests phage can adopt different conformations allowing monovalent or multivalent interactions with the bacterial receptors. This has been demonstrated in binding experiments in which β-galactosidase from *E.coli* served as a model multivalent analyte (Petrenko and Vodyanoy, 2003). Results of bacteria- and phage-capture ELISAs may differ also because the anti-fd phage antibodies used for revealing of cell-bound phage in the phage-capture assay can bind unequally with different recombinant phage clones.

Representative clones demonstrating ELISA signals higher than control wild-type phage were characterized by a precipitation assay to confirm their specificity for *Salmonella.* This assay, previously described for the analysis of phage binding to zoospores and bacterial spores (Bishop-Hurley et al., 2002; Knurr et al., 2003), was optimized to avoid aggregation and self-precipitation of phage (Brigati et al., 2004). Using this assay we found that yields of *Salmonella*-bound phages were 12,000-22,000 times higher than the yield of the control wild-type phage, indicating very high specificity of the selection procedure.

Phage VTPPTQHQ demonstrating the highest binding to the bacterium was studied in more detail to estimate affinity and selectivity of its binding to *Salmonella*. Dose-dependent binding of phage to cells in solution was observed in phage concentrations ranging from 10^1 to 10^{12} CFU/ml (Fig. 1A) with maximum yield of bound phage $\sim 5 \times 10^7$ CFU ($\sim 10^9$ vir), comparable or exceeding the number of bacterial cells involved in the complex formation ($\sim 10^8$). Indeed, the TEM images of bacteria-complexed phage clearly show that more than one phage particles can bind to one bacterial cell (Petrenko and Sorokulova, 2004). The highest recovery of bound phage (15%), measured as a ratio of output to input phage, was obtained when equal or surpassing amounts of bacteria were involved in the complex formation (10^6- 10^8 CFU/ml phage *versus* $\approx 10^8$ CFU/ml *S. typhimurium*) (Fig. 1B). The K_d of the complex calculated from the Hill plot (Fig. 1C) is 4.77×10^7 CFU/ml ($K_{d(apparent)} = 3.18 \cdot 10^9$ CFU/ml), corresponding to 1.6 pM of phage, or 6.3 nM of the phage-borne peptide ($K_{d(apparent)}$ = 106 pM phage and 422 nM peptide) . The extraordinary high affinity of phage binding may be attributed to an avidity effect of the multivalent interaction of phage, carrying 4,000 binding peptides, with multiple bacterial surface receptors. The multivalent interaction of phage with cells appears to be confirmed by TEM data (Petrenko and Sorokulova, 2004). Hill coefficient (n = 0.81) estimated from Hill plot (Fig. 1C) also indicates that one phage particle may bind to more

then one bacterial cell receptor. The affinity data of phage-cell interaction, however, should be considered with prejudice because we cannot exclude that bacterial cell receptors are not equally competent to phage binding.

Binding of phage VTPPTQHQ to *Salmonella* was also confirmed by fluorescence-activated cell sorting, and visualized by fluorescent microscopy, transmition and scanning electron microscopy. The phage was fluorescently labeled by Alexa Fluor 488 (Molecular Probes) with a density of 300 dye molecules per phage. Fluorescently labeled phage (10^9 CFU) was incubated with *Salmonella* cells at room temperature for 1 hour and centrifuged at 3,500 rpm, as in the precipitation test. The complex of phage with bacteria was detected by FACS (Petrenko and Sorokulova, 2004) and visualized by fluorescence microscopy (not shown here).

We also observed binding of *Salmonella* to the immobilized phage directly, by high power optical microscopy and scanning electron microscopy. In this test, small gold chips were coated with the selected test phage and an unrelated control phage using chemisorptions and/or hydrophobic absorption. The chips were soaked in a suspension of *Salmonella* cells, washed and analyzed microscopically. Using this test we observed in real time specific binding of bacterial cells to the immobilized phage (Fig. 2), and we confirmed this binding by scanning electron microscopy (Fig. 3).

3.3. Selective binding of phage to S. typhimurium

We define selectivity as the ability of a recombinant phage clone to preferentially interact with a select target, i.e. bacteria, in comparison to other potential targets. Binding of the phage probes with *S. typhimurium* was compared with their binding to control strains of other gram-negative bacteria, predominately *Enterobacteriaceae,* chosen for their phylogenic relatedness to *S. typhimurium.* In the co-precipitation assay, each organism was incubated with an equal amount of the phage

VTPPTQHQ, and bound virions, recovered by lysis of bacteria with DOC buffer, were titered by infecting *E. coli* K91BK host cells. Precipitation assay demonstrated 10-1000 times greater binding of phage to *S. typhimurium* versus the challenge bacteria (Fig. 4).

4. Discussion

Monitoring of food and environmental samples for biological threats, such as *S. typhimurium*, requires probes that specifically bind biological agents and insure their separation, purification and detection (Mansfield and Forsythe, 2000; Petrenko and Sorokulova, 2004). These binding probes can be isolated from diverse peptide or antibody libraries using phage display (Petrenko and Vodyanoy, 2003). The prospect of this technology has been proven by the development of probes for various bacterial, viral and toxic agents (Petrenko and Sorokulova, 2004). While the peptides and antibodies discovered through phage display are valuable probes for laboratory assays, landscape phage probes may be better suited for food and field monitoring, where robust inexpensive probes and biosorbents are needed. These probes may be recruited from diverse libraries of landscape phages originated from the filamentous phage fd (Petrenko and Smith, 2000; Petrenko et al., 1996). Like the parental phage fd, which is probably the most stable natural nucleoprotein withstanding high temperatures, denaturing agents, organic solvents, mild acids, and alkaline solutions, the phage-derived probes, burdened by thousands of guest peptides, inherit the extreme robustness of the wild-type phage. Furthermore, phage is an inexpensive standard construction material that allows fabrication of bioselective layers by self-assemblage of virions or their composites on solid surfaces (Petrenko and Vodyanoy, 2003). .

The landscape phage library contains many potential probes for surface markers of *S. typhimurium*, which can be isolated by single- or multistage selection using immobilized or suspended bacteria as a selector. In this study, we elaborated three different selection procedures,

resulting in discovery of five families of bacteria-binding peptides (Table 4). One feature that stands out among these peptides is the high amount of proline, which is known to dominate in some antibacterial peptides (Cudic et al., 2002). Specificity of isolated phage clones for *S. typhimurium* was demonstrated using two variations of ELISA (phage capture and *Salmonella* capture) and confirmed by precipitation assay, fluorescence and electron microscopy. We also compared binding of representative clone VTPPTQHQ to *S. typhimurium* and a panel of select bacteria, primarily *Enterobacteriaceae*, revealing high selectivity of the phage to the selector bacterial strain. However, greater cross reactivity was noted with *Y. enterocolitica* and *C. freundii* that other challenge bacteria. It was not surprising since we used non-biased selection conditions allowing phages from the diverse landscape library to interact with any one of numerous surface receptors of *S. typhimurium*, including those that may be common for the panel strains.

In fact, the strains from the family *Enterobacteriaceae* are highly similar in their surface structure. The major components of their outer membrane include the lipopolysaccharide (LPS), phospholipids, proteins and a unique cell surface glycolipid--their common antigen (Rick, 1996). In gram-negative bacteria, composition of LPS is highly variable between genera, species and even strains, differing in a number and structure of repeating oligosaccharide units, known as O-antigens or O-chains, which are linked to lipid A via a genera-specific core polysaccharide. These differences account for serotype specificity; to date, well over 40 major serogroups in *Salmonella* species, over 170 serotypes in *E. coli*, greater than 40 in *Shigella* spp., *Citrobacter* spp., and *Proteus* spp., and greater than 10 in *Klebsiella* spp have been identified (Weintraub, 2003). LPS has been shown to serve as receptors for some natural strain-specific phages (Romanowska et al., 1976) and may serve as a target for landscape phages. If it is true, then some cross-reactivity of *S. typhimurium*-targeting phage with *C. freundii* and *Y. enterocolitica* may be attributed to identical or

near identical monosaccharide and disaccharide O-antigen components, which have been noted between *Salmonella* and *Citrobacter* species (Knirel et al., 2002).

Numerous highly specialized, variable major and minor proteins, interlaced among the LPS, may be also plausible targets for landscape phages. They serve common functions among the gram-negatives including selective and nonselective translocation and structural integrity, e.g. murine-lipoprotein and OmpA (Benz and Bauer, 1988), but may vary in primary structure. Major proteins of the gram-negative bacteria in general include beta-barrel OM porins such as OmpF, OmpC, OmpD, PhoE, Oms1, OmpN, NmpC, and OprP, which allow *nonspecific*, hydrophilic, low molecular weight substances to diffuse across the membrane by size exclusion, and *specific* porin receptors that bind particular extracellular target substances via molecular recognition such as LamB (maltodextrines, mono- and disaccharides), Tsx (nucleotides), ScrY (saccharose), OprB (phosphate), or OprO (*P. aeruginosa* – pyrophosphate). Minor proteins include iron transporters such as FhuB, and those that function in vitamin uptake.

In our experiments, prior to affinity selection, the phage library was propagated in *Escherichia coli* cells, which may result in elimination of clones specific for *E. coli*-surface receptors. Therefore, it is reasonable to hypothesize that the phage-targeted receptor(s) of on the surface of *S. typhimurium* differ from *E. coli* receptors. A comparison of OmpC, OmpF and NmpC porins between the two strains has shown that only half of the proteins are similar or identical (Lee and Schnaitman, 1980). For example, N-acetyl phenylalanine β-naphthyl esterase (ApeE) has been demonstrated in *S. typhimurium* but not *E. coli* (Carinato et al., 1998); whereas, OmpA, – a phage receptor of *E. coli* (Osborn and Wu, 1980), has not been demonstrated in *S. typhimurium*.

Finally, there exist several outer membrane associated proteinaceous structures such as fimbriae, and in some genera, pili and flagella, which are antigenic and highly variable between

genera. In particular, fimbriae are hair-like appendages composed of protein subunits called fimbrins, which extend outward from the bacterial surface and may function as an adhesion factor for specific receptors on target cells. Fimbriae are prevalent on *S. typhimurium* as well as other enteric bacteria; multiple types can be expressed by a single isolate. Assuming that the fimbriae are the source of phage affinity for *S. typhimurium,* cross reactivity of the phage with *Salmonella* and *Citrobacter* in precipitation assay may be explained by a distinct serological relationship between their Type I fimbriae (Nowotarska and Mulczyk, 1977). Any fimbrial relationship between *S. typhimurium* and *Y. enterocolitica* is unknown. Thus, while the outer membrane-associated structures are common to gram-negative bacteria, and in particular the enteric bacteria, the potential structural dissimilarity in the components between genera lends itself to explain the selective binding *S. typhimurium* exhibited by the isolated landscape phage. Identification of the phage-targeting structures on the surface of bacterial cell would allow a clear explanation of phage specificity and selectivity.

Landscape phages discovered in this work may find application as biosorbents and diagnostic probes for monitoring of *Salmonella* by various devices in which antibodies or peptides have been used. For example, they may be used for separation and purification of bacteria prior to their identification with polymerase chain reaction, immunoassays, flow cytometry, or other methods. Furthermore, they may find application as biorecognition elements of real-time biosensor devices. We recognize that described probes are not completely ideal for identification of *S. typhimurium* because they cross-react with another enterobacteria. However, the power of phage display technology allows control of the phage selectivity by engineering the biased selection schemes (Petrenko and Sorokulova, 2004). Thus, cross-reacting clones may be removed from the library by its prior depletion against competing strains, as was previously demonstrated (Petrenko

and Sorokulova, 2004). The depleted library will serve as a reservoir of probes for unique *S. typhimurium* markers. If necessary, the affinity of probes may be also increased by directed molecular evolution, including iterative mutagenesis of phage and selection of the bacterial-binders in more stringent conditions (Petrenko and Sorokulova, 2004). Therefore, it is our contention that performance of the selected phage-derived probes can be gradually enhanced through biased selection strategies and directed molecular evolution. We believe that this new generation of robust and inexpensive phage-derived probes will serve as efficient substitutes for antibodies in separation, concentration and detection systems employed for food and environmental monitoring.

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Figure Legends

Fig. 1. Binding of phage VTPPTQHQ to *S. typhimurium* as demonstrated by coprecipitation-titration assay.

A. Concentration-dependent binding isotherm of phage to *S. typhimurium*. Smooth line is B-spline curve fit to data points. Bars = SD.

B. Mean values (n = 3) of yield (output /input, %) are plotted as a function of mean input concentration of phage VTPPTQHQ.

C. Hill plot of binding isotherm. The ratio of bound and free *S. typhimurium* receptors is shown as a function of phage VTPPTQHQ concentration. Line represents the linear least squares fit of experimental data ($R = 0.967$, $SD = 0.612$, $p = <0.001$). Hill coefficient, $n = 0.81$, and association binding constant ($K_b = 2.1 \cdot 10^{-8}$ CFU/ml; $K_{d(apparent} = 3.18 \cdot 10^{9}$ CFU/ml), were derived from the plot. Explanation of the Hill plot is provided in subsection 2.6.

Fig. 2. Interaction of *S. typhimurium* ATCC 13311 with the phages, attached to the gold surface: A – non related phage EAGPRSAP; B – selected phage VTPPTQHQ.

Fig. 3. Study of interaction of *S. typhimurium* ATCC 13311 with selected phage VTPPTQHQ by scanning electron microscopy: A – plain gold surface nontreated with phage and bacteria; B -- gold surface, coated with phage and treated with bacteria (the arrow shows an attached *Salmonella* cell).

Fig. 4. Selectivity of phage VTPPTQHQ as determined by coprecipitation assay. Mean yield (output/input x 100) percentage is the average of three separate experiments normalized to the maximal mean yield of 3.4% from *S. typhimurium* (1). Numbers above bars indicate respective

percentages. (2): *Pseudomonas aeruginosa;* (3): *Enterobacter aerogenes;* (4): *Citrobacter freundii;* (5): *Klebsiella pneumoniae;* (6): *Shigella flexneri*; (7): *Escherichia coli;* (8): *Yersinia enterocolitica;* (9): *Serratia marcescens;* (10): *Proteus mirabilis*.

Tables

Table 1

Solutions, reagents, preparations and media

Solution or preparation	Description and preparation procedure
Alkaline phosphatase conjugated goat anti-rabbit antibody IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), diluted 1:5000 in TBS/0.5% Tween
AP-SA	Alkaline-phosphatase-conjugated streptavidin; Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) 016-050-084; dissolved at a concentration of 500 µg/ml in 5 mM Tris-HCl (pH 8), 125 mM NaCl, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 50% (v/v) glycerol; stored at 4 °C
AP-SA diluent	1 mg/ml BSA, 0.1% Tween 20, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ in TBS
Binding buffer	1% BSA, 0.5% Tween in TBS, filter sterilized
BSA	Bovine serum albumin, Fraction V; Sigma Chemical Co. (Saint Louis, MO) A2153; 50 mg/ml stock is filter-sterilized and stored at 4 °C
BSA covered plates	Add to every Petri dish (35 mm) 1 ml of 1 mg/ml BSA in TBS and keep overnight at 4 °C
DOC lysis buffer	2% sodium deoxycholate, 10mM Tris, 2mM EDTA, pH adjusted to 8.0 with HCl; filter-sterilized

λ DNA markers	*Bst*EII digest of phage λ DNA: 8453, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, 224 and 117 base pairs (bp)
dNTP's	Mixture of equal concentrations of dATP, dGTP, dCTP and dTTP
Elution buffer	0.1 N HCl, 1 mg/ml BSA, pH adjusted to 2.2 with glycine; made by mixing water, 50 mg/ml BSA, and 0.4 N HCl, pH adjusted to 2.2 with glycine; filter-sterilized and stored at room temperature
40· GBB	1.68 M Tris, 0.8 M sodium acetate, 72 mM Na_2EDTA, pH adjusted to 8.3 with glacial acid
Incubation buffer for electron microscopy	PBS, 0.1% BSA-C (Aurion, Electron Microscopy Sciences, Hatfield, PA), 20 mM NaN_3, pH 7.4
NAP buffer	80 mM NaCl, 50 mM $NH_4H_2PO_4$, pH adjusted to 7.0 with NH_4OH; autoclave and store in a refrigerator or at room temperature
NZY broth	Dissolve 10 g NZ amine A, 5 g yeast extract, and 5 g NaCl in 1 liter water; adjust pH to 7.5 with NaOH; autoclave; store at room temperature
NZY plates	Autoclave 11 g Bacto-agar in 500 ml water in a 2-liter polypropylene flask; without cooling, add 500 ml 2 · NZY medium at room temperature; add supplements such as tetracycline as required; swirl to mix; pour about 25 ml per 100-mm Petri dish.
PBS	0.15 M NaCl, 5 mM NaH_2PO_4, pH 7.0 adjusted with NaOH; autoclave
PEG/NaCl	100 g PEG 8000, 116.9 g NaCl, 475 ml water; autoclave
Salmonella O Poly A-1 & Vi Antiserum	Becton-Dickinson and Company (Franklin Lakes, NJ), diluted 1:500 in TBS/0.5% Tween

p-Nitrophenyl Phosphate tablets (NPP)	Sigma (Saint Louis, MO), 5 mg substrate per tablet
NPP diluent	For 1 tablet : 5 ml 1M diethanolamine buffer (pH 9.8), 5 µl 1M $MgCl_2$
TBS	50 mM Tris-HCl (pH 7.5), 0.15 M NaCl; autoclave
TBS/Tween (washing buffer)	0.5% (v/v) Tween 20 in TBS; filter-sterilized

Table 2

Differences in selection procedures

Procedure	Separation of bacteria/phage complexes	Blocking, BSA (%)	Library depletion	Wash, Tween (%)	Rounds of selection
A	Immobilization	0.1	NO	0.1	5
B	Immobilization	0.1	Plastic, BSA-coated plastic	0.5	4
C	Precipitation	0	Centrifugation	0.5	3

Table 3

Recovery of *S. typhimurium*-binding phages during selection

Procedure[a]	Yield (output/input), % / round of selection				
	1	2	3	4	5
Selection A					
Acid fraction	1.1×10^{-2}	2.4×10^{-3}	1.1×10^{-2}	3.8×10^{-2}	1.6×10^{-1}
DOC fraction	9.1×10^{-3}	8.8×10^{-3}	8.6×10^{-3}	2.3×10^{-2}	4.7×10^{-2}
Selection B					
Acid fraction	8.6×10^{-3}	1×10^{-2}	1.5×10^{-1}	1.3×10^{-1}	
DOC fraction	6.3×10^{-3}	2.2×10^{-5}	3.1×10^{-5}	1.3×10^{-4}	
Selection C					
Acid fraction	2.4×10^{-4}	3.1×10^{-3}	3×10^{-2}		
DOC fraction	0	0	0		

[a]Three different protocols were used for selection of the *Salmonella*-binding phages: A, B and C, as described in Methods, subsections 2.4.1, 2.4.2 and 2.4.3.

Table 4

Phages isolated from the landscape phage library f8/8 by affinity selection with *S. typhimurium* cells

	Acid fraction				DOC fraction			
	Family	Phage-borne peptide[c]	ELISA[a] PC	ELISA[a] SC	Family	Phage-borne peptide[c]	ELISA[a] PC	ELISA[a] SC
A[b]	I	VTPPTQHQ[4]	45.0	26.6	III	ERPPNPSS[8]	32.1	24.4
	I	VTPPSQHA[10]	55.2	25.7	III	ERSSQANM	N/A	N/A
	I	VSPPPQHS	63.9	30.5	III	ERTTSAHT	N/A	N/A
	I	VSPQSAPP	44.9	39.6	III	DRTSNQAT	N/A	N/A
					III	DLTSNQAT	N/A	N/A
					I	VSPPSNPS[2]	11.4	33.8
					I	VTPPSQHA		
B[b]	I	VTPPQSSS	N/A	43.5	I	VTPPQQGS	N/A	14.1
	I	VTPPTSPQ	N/A	34.6	IV	DPRSPASL[2]	N/A	22.9
	I	VTPSSPHS	N/A	33.4	IV	DPRPAQHT	N/A	16.7
	I	VTPQGSHP	N/A	36.3	IV	DPHKAGGL	N/A	20.2
	I	TPGQPSHP	N/A	28.3	IV	DPKSPLHT	N/A	33.4
	I	VSTQSTHP	N/A	40.9	IV	DPKSPQQT	N/A	63.4
	II	VPPPSPQS[2]	N/A	28.3	IV	DPKGPHSM	N/A	14.8
	II	VPPPSPHS[3]	N/A	4.3	IV	EPHRAASV	N/A	19.6
	II	VPPPSASS	N/A	10.3	IV	EPRLAHGA	N/A	19.5
	II	VPPPSQSQ[2]	N/A	16.5	IV	DNKMTSHS	N/A	19.5
	II	VPPPSNPS	N/A	18.1	V	DPSKRTQP	N/A	37.6
	II	VPPPGQHQ	N/A	0.2	V	EPNKHSQS	N/A	27.9
	II	VPPSSSSP	N/A	31.6	III	DRPSPNTV	N/A	42.1
		VPQQDKAQ	N/A	60.6				
C[b]	I	VSSNQAPP[18]	N/A	25.9				
	II	VPIPYNGE	N/A	10.0				
	III	DRSPSSPT[4]	N/A	29.2				

[a]Enzyme linked immunosorbent assay (ELISA) was carried out in two formats: phage-capture ELISA (PC) and *Salmonella*-capture ELISA (SC), as described in Methods, subsections 2.10 and 2.11. ELISA signals are expressed in OD/1000 min (mOD/min). The background binding signal of wild-type vector f8-5 (17.7 mOD/min) is deducted. NA - phages that were not analyzed by ELISA.

[b]Three different protocols were used for selection of the *Salmonella*-binding phages: A, B and C, as described in Methods, subsections 2.4.1, 2.4.2 and 2.4.3.

[c]Families of homologous peptides are designated by Rome numbers. Common motives are indicated by bold letters. Superscripted Arabic numbers show how many identical clones were identified in the group of the sequences phages.

Figure 1A.

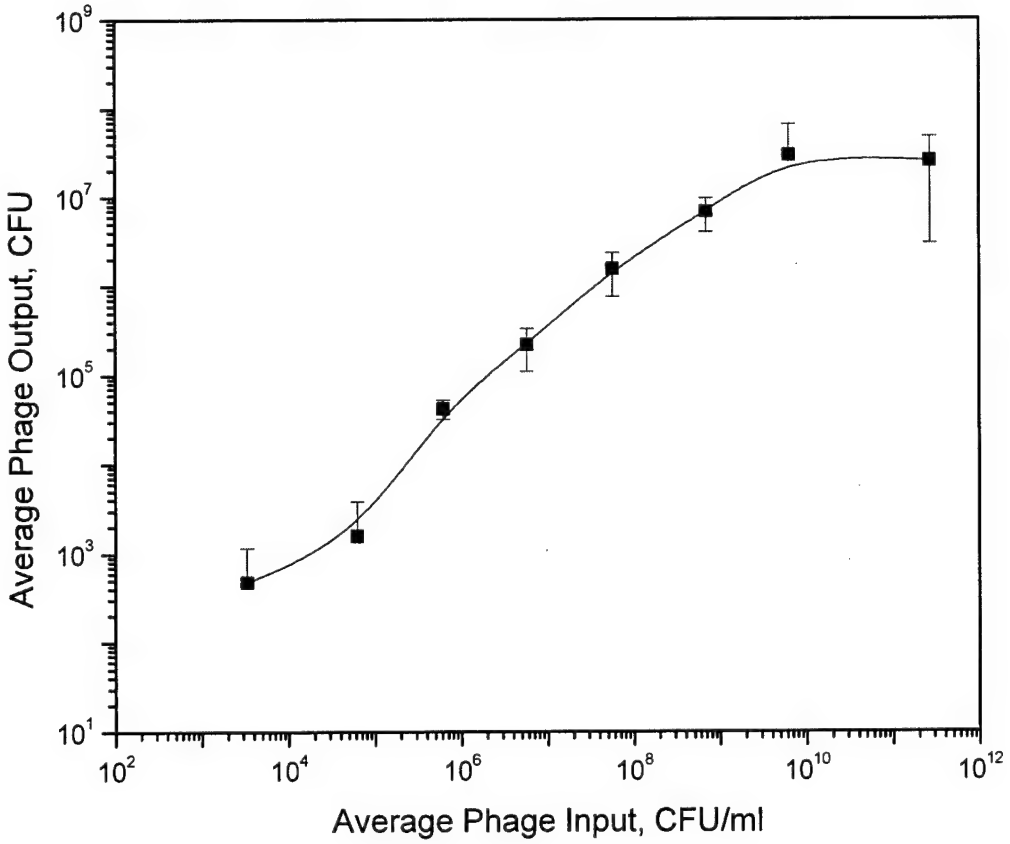

Average Phage Output, CFU
10^9
10^7
10^5
10^3
10^1
10^2
10^4
10^6
10^8
10^{10}
10^{12}
Average Phage Input, CFU/ml

Figure 1B.

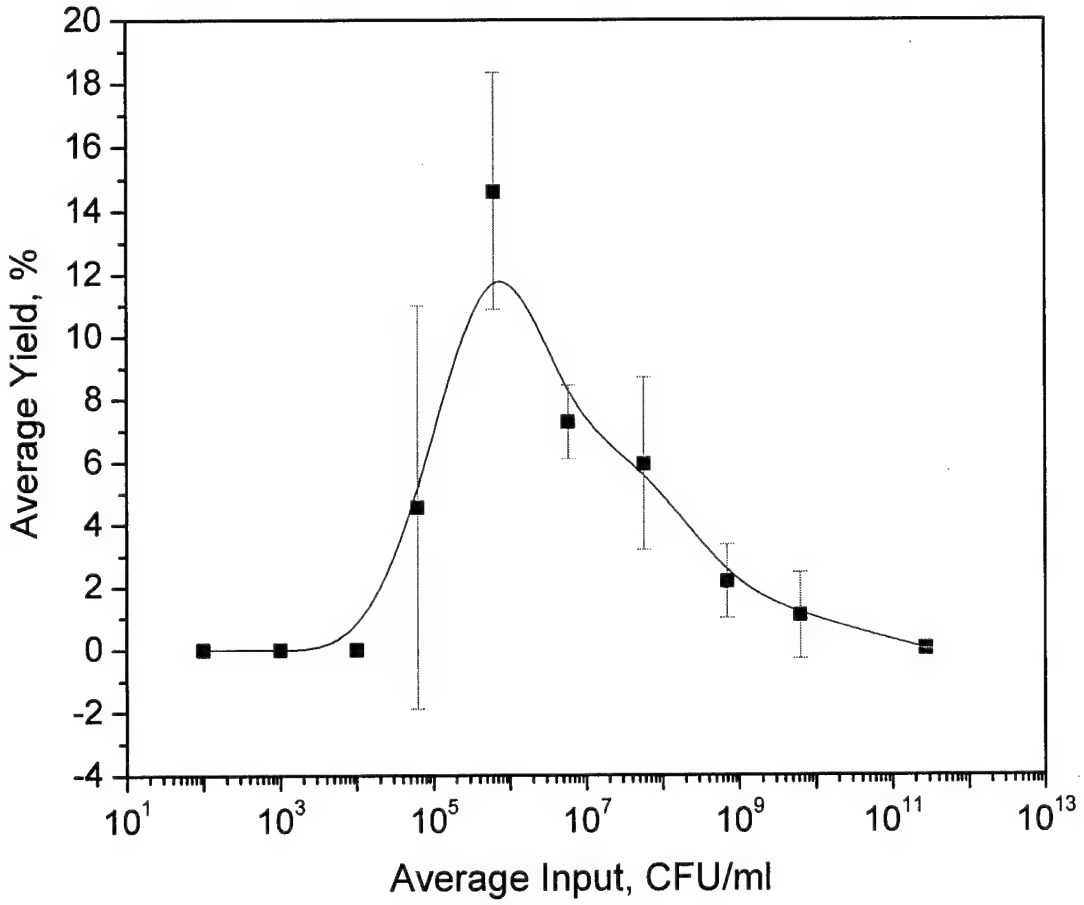

Average Yield, %
20
18
16
14
12
10
8
6
4
2
0
-2
-4
10^1
10^3
10^5
10^7
10^9
10^{11}
10^{13}
Average Input, CFU/ml

Figure 1C.

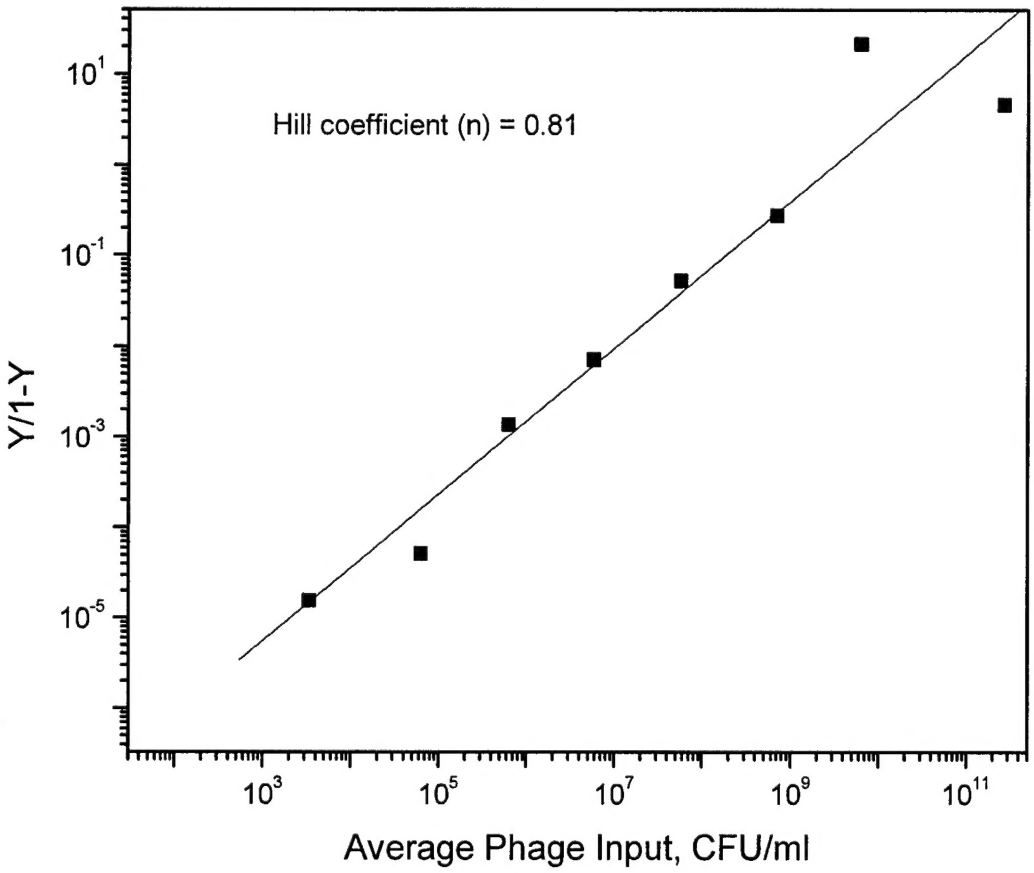

Hill coefficient (n) = 0.81
Y/1-Y
10^{1}
10^{-1}
10^{-3}
10^{-5}
10^{3}
10^{5}
10^{7}
10^{9}
10^{11}
Average Phage Input, CFU/ml

Figure 2.

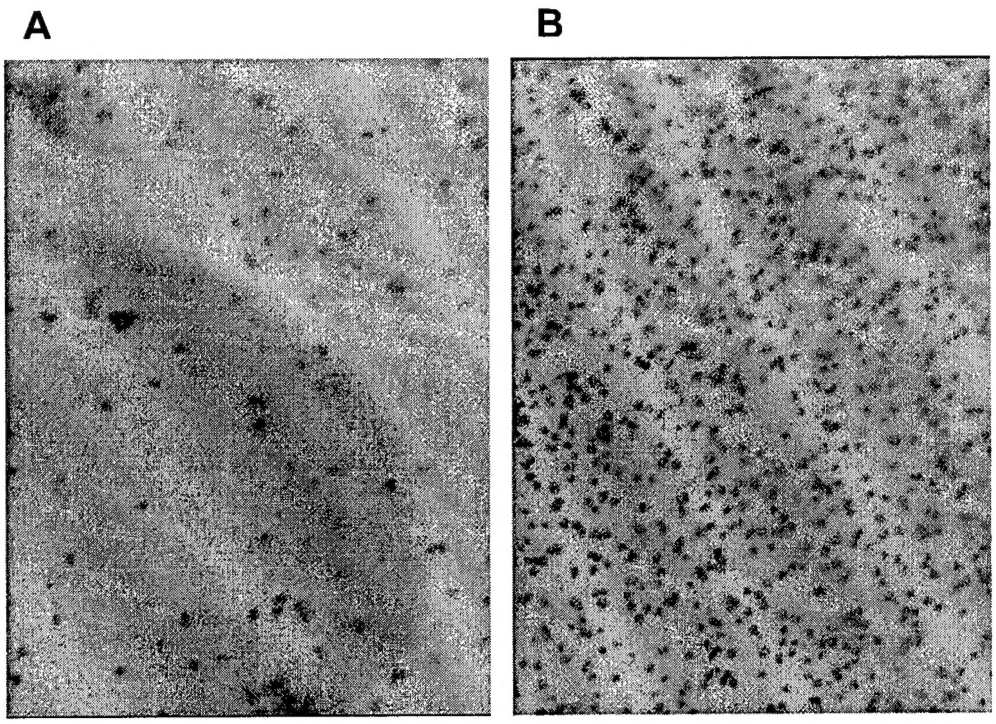


Figure 3.

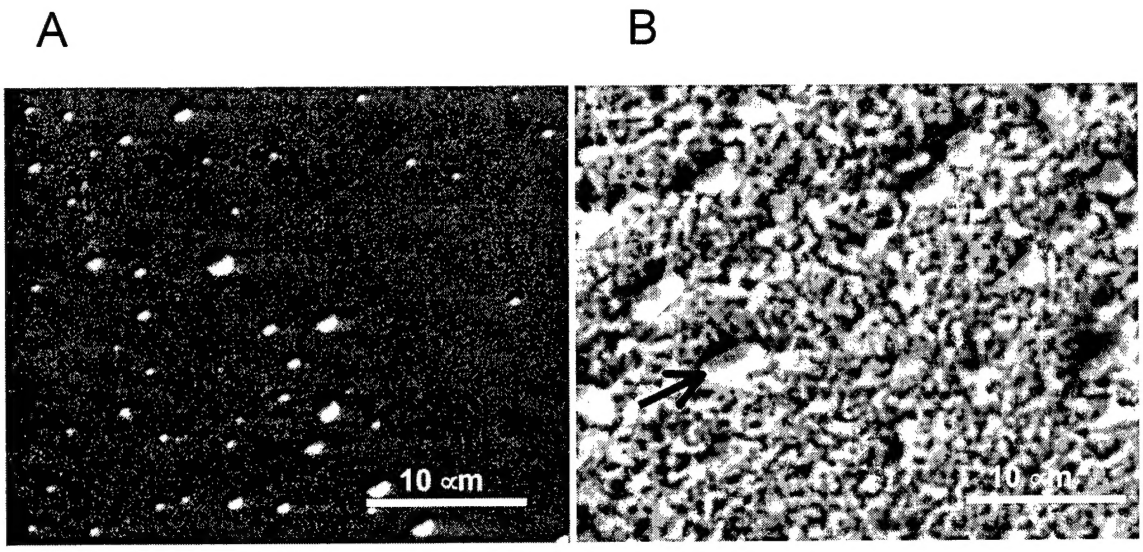
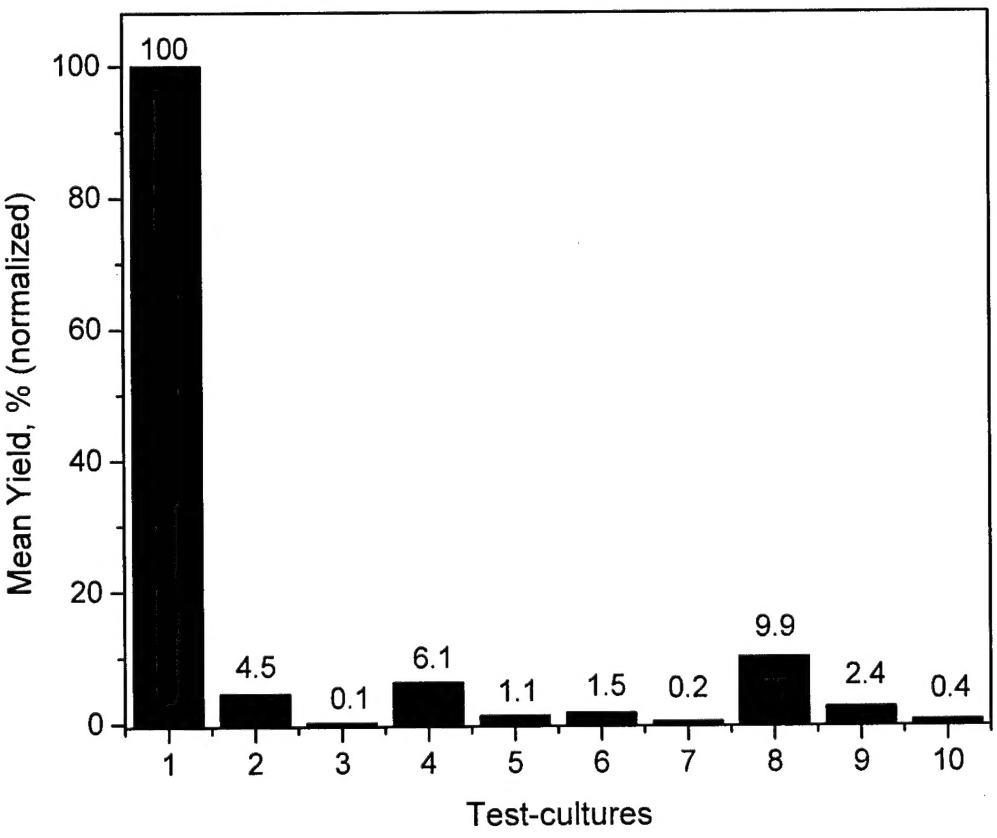


Figure 4.


Mean Yield, % (normalized)
Test-cultures
100
4.5
0.1
6.1
1.1
1.5
0.2
9.9
2.4
0.4
0
20
40
60
80
100
1
2
3
4
5
6
7
8
9
10